Solid-Phase Extraction for Sample Preparation, in the HPLC Analysis of Polyphenolic Compounds in "Fino" Sherry Wine

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Wine samples are extremely complex in their content of polyphenolic compounds; this necessitates an initial fractionation prior to their analysis by HPLC, to simplify the chromatograms obtained so that they can be reliably identified and quantified. Solid-phase extraction is one of the appropriate techniques for this purpose. This paper presents the work undertaken to devise a method of fractionating polyphenolic compounds in wine; this comprises a sequence of two stages: first, one of cleaning and preconcentration, using a reversed-phase C18 cartridge, and second, one of fractionation into two groups, acid and neutral polyphenols, using a SAX-type anionic exchanger. Both of these stages have been optimized, using standard solutions, to achieve maximum recovery and reproducibility.

Keywords: *Solid-phase extraction (SPE); HPLC; polyphenols; wine*

INTRODUCTION

The fact that polyphenolic species are involved in the "browning" phenomenon in white wines is widely known and accepted (Singleton and Esau, 1969). This phenomenon has a direct and adverse effect on the quality of these wines, so justifying the importance of studying it and developing analytical methods to enable the identification and quantification of the compounds implicated, to follow their evolution from the grape to the finished wine. Since the sample is highly complex, the analysis of polyphenols in wine calls for a preconcentration and fractionation stages prior to the employment of analytical techniques such as HPLC, to simplify the extremely complicated chromatograms.

One of the most frequently used techniques for the preparation of wine samples is liquid−liquid extraction with ethyl ether (Fernández de Simón et al., 1990), but because this lacks selectivity, very complex chromatograms still result. Another difficulty related to the use of this technique concerns the number of analytical artifacts which can appear, as the authors have already pointed out (Bru et al., 1996). Others writing on this topic have used extraction with ethyl acetate, adjusting the pH to 7 and 2 to separate the polyphenolic species into two groups, but even with this method, the chromatograms obtained are still very complex (Salagoity-Aguste and Bertrand, 1984; Di Stefano and Garcia-Moruno, 1986). Recently, however, the technique of extraction over solid adsorbents has been applied. In the Literature Cited there are already many papers applying this technique for the preparation of samples prior to HPLC analysis of polyphenolic species in various foods (Kim and Keeney, 1983; Seo and Morr, 1984; Papadopoulos and Tsimidou, 1992), in grapes (Lee and Jaworski, 1989), and in musts and wines (Jaworsky and Lee, 1987; Ozmianski et al., 1988; Cartoni et al. 1991). But what is basically carried out in all these cases is a retention over C18 and a fractionation eluting with solvents at different pH levels.

Presented in this paper is a description of the research undertaken to devise a fractionation scheme for polyphenolic compounds in "fino" sherry wine by solid-phase extraction, involving a first stage of preconcentration and cleaning of the sample through an adsorbent C18 and a second stage of fractionation using a SAX anionic exchanger.

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MATERIALS AND METHODS

The operating conditions were established using mixtures of phenolic acids and aldehydes from Fluka (Buchs, Switzerland) and Eastman Kodak (Rochester, NY), dissolved in a matrix medium containing tartaric acid (3 g/L) and ethanol (15% v/v), both of analytical reagent grade, from Merck (Darmstadt, Germany), in concentrations similar to those described in real samples of wine (Table 1).

The samples were passed through two solid-phase extraction cartridges, first a Bond-Elut C18, then a SAX with 500 mg of filling from Analytichem International (Varian, Harbour, CA), by means of a SPE vacuum device from Supelco (Bellefonte, PA) which allows 12 samples to be handled simultaneously.

Finally, the samples obtained were analyzed by HPLC using a Waters chromatograph (Milford, MA) comprising two pumps (a model 6000A and a model M-45), a model U6K injector, a model M490 detector, and a Millenium 2010 computer chromatographic data station to control the system. The chromatographic separation was carried out using a Lichrospher-100 steel cartridge column of 25 cm length, 4 mm i.d. with 5 μm particle size from Merck (Darmstadt, Germany), and the gradient worked out by the authors (Guillén et al., 1993).

RESULTS AND DISCUSSION

To optimize the sample preparation stage, our strategy was based on an understanding of how those phenolic compounds of enological interest fall into families or groups when present in "fino" sherry wine. It is known that, in terms of variety and quantity, most of the phenolic compounds present comprise the acids as one group and a second group of neutral compounds represented by the phenolic aldehydes and the catechines. Consequently, the fundamental objective of this work was to achieve a fractionation into these two families.

Initially, some preliminary experiments were done to observe the behavior of the polyphenolic species through the solid adsorbents as a function of different variables

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Table 1. Composition of the Mixed Solution of Standards

compd	concn (mg/L)
gallic acid	4.24
protocatechuic acid	5.40
protocatequialdehyde	2.88
gentisic acid	33.40
p-hydroxybenzoic acid	7.32
catechin	17.26
p-hydroxybenzaldehyde	1.92
m-hydroxybenzoic acid	5.80
vanillic acid	2.42
caffeic acid	2.44
syringic acid	2.32
vanillin	6.72
epicatechin	18.1
syringialdehyde	3.20
p-coumaric acid	1.72
o-vanillin	6.28
ferulic acid	2.80
veratric acid	13.56
sinapic acid	6.28
o-coumaric acid	1.52
3,4,5-trimethoxycinnamic acid	2.12
3,5-dimethoxybenzaldehyde	5.20

of the technique. The first attempt studied was the possibility of achieving a fractionation by the addition of an ion pair reagent and the subsequent fractionation through a C18 adsorbent where the phenolic acids would be retained longer, while the neutral species would be eluted normally. No significant conclusion could be drawn from these experiments, except confirmation that the retention of the acids was more influenced by mechanisms of ionic suppression than by the formation of the ion pair itself.

A further study was made to check the possibility of achieving a fractionation over a C18 adsorbent using an ionic suppression mechanism; however, when the fractions collected were injected into the HPLC, it was clear that they had not divided cleanly into the two families.

Having proved the impossibility of getting a clean fractionation into acid and neutral polyphenols by the methods tried, the alternative technique of ionic exchange was selected for experiment. The adsorbent chosen to study the fractionation of polyphenols into families was the SAX strong anionic exchanger.

Initially, a scheme was planned for the elution over the SAX cartridge selecting the pH level of the medium in which the sample was going to be injected as a function of the average pK_a of the phenolic acids. The experiments were carried out with model solutions of acid and neutral phenolic internal standards. On the basis of the results achieved, modifications to the initially planned scheme were introduced, changing the compositions and volumes of the solutions used for the separate stages of the process—injection, washing, and elution—the objective of these modifications always being the achievement of the longest possible retention time for the acid species through the exchanger while at the same time trying to ensure that the neutral species were not retained. The best results were obtained under the conditions described in Table 2.

As has been already mentioned in the Introduction, the levels at which phenolic compounds are present in "fino" sherry wine are relatively low. Since the requirements of the ionic exchange technique demand that excessive volumes should not be injected, it is necessary to provide for a prior stage of preconcentration. Furthermore, there are high concentrations of anions such as tartrate, bitartrate, malate, succinate, etc., in the wine; these are obviously disposed to compete with the phenolic acids for the site of the exchanger, with the result that the retention time of the acids over the adsorbent is reduced. Consequently, this factor reinforces the indicated need to introduce a cleaning stage to eliminate these possible interferences, prior to the stage of fractionation over the exchanger.

The ideal situation would be to carry out the cleaning of the sample at the same time as a preconcentration of the phenolic species. The method selected for this stage was a solid-phase extraction using C18 as adsorbent since this should, a priori, given its characteristics and under appropriate conditions, be capable of selectively retaining the phenolic species but not the interfering substances. This, together with the subsequent elution of the retained species using only a small volume of solvent, should enable the two planned objectives to be achieved.

Following a process similar to that of the preceding section, to select the best operating conditions for this stage, an initial working plan was proposed. Then, on the basis of the results, modifications to the plan were introduced, changing the compositions and volumes of the solutions used in the separate stages of the process—injection, washing, and elution—all with the aim of obtaining the maximum retention time for all the polyphenolic species under study. The best results were achieved under the conditions described in Table 3.

Once the conditions for both stages had been optimized individually, the next step consisted of checking the effectiveness of the overall scheme. An experiment was carried out, with the solution of internal standards, in which the two stages were applied sequentially; good performance was observed in the cleaning and preconcentration stage, but the results obtained in the fractionation stage were not acceptable. The cause of the poor fractionation obtained in the exchanger could have been due to the presence of methanol in the solution injected into the exchanger, given that the experiments performed up to then to test the efficiency of of this stage had been done without the presence of this solvent.

A modification was made which consisted of reducing from 1.5 to 0.5 mL the volume of methanol used to elute the phenolic compounds from the C18; in this way the proportion of methanol in the solution subsequently injected into the SAX was reduced. This was found to improve the results considerably.

In conclusion, the best overall scheme for the preparation of samples by SPE is summed up in Table 4.

A study was carried out on the reproducibility of the sample preparation method, repeating the process 12 times, using the solution of standards in the matrix medium (15% v/v ethanol, 3 g/L of tartaric acid, in water) as sample. The results obtained are presented in Table 5. Here, it can be seen that the recovery rate of most of the species, with the exception of gallic acid, protocatechuic acid, and protocatequialdehyde, is above 50% and the relative standard deviation ranges from 1.94% to 22.69%; this represents an improvement over other sample preparation techniques such as liquid–liquid extraction. Furthermore, it should be pointed out that this method shortens the analysis time by approximately 50% and reduces to one-tenth the volume of sample required for the analysis.

Illustrated in Figure 1 are the chromatograms resulting from the application of the fractionation process devised here to a sample of "fino" sherry wine. These

Table 2. Operating Conditions for Fractionation Stage

adsorbent	SAX (500 mg) equilibrated with 10 mL of water	
sample	3 mL solution of standards buffered to a pH of 6.5, with phosphate buffer of $I = 0.05$ M	the eluate in which the neutral polyphenols should be present is collected in this step
washing solvent	1 mL of water	
elution	1 mL of 1 M HCl	this eluate corresponds to the acid polyphenol fraction

Table 3. Operating Conditions for Cleaning and Preconcentration Stage

adsorbent	C18 (500 mg) solvated with 10 mL of methanol conditioned with 3 mL of the saturated NaCl solution at a pH of 2 (25 g of NaCl in 100 mL of 0.01 M HCl)	
sample	5 mL solution of standards diluted with 5 mL of a saturated solution of NaCl at a pH of 2 (25 g of NaCl in 100 mL of 0.01 M HCl)	
washing solvent	1°: 1 mL of the saturated NaCl solution at a pH of 2 (25 g of NaCl in 100 mL of 0.01 M HCl) 2°: 1 mL of a solution of 0.01 M HCl	
elution	1.5 mL of methanol	this eluate corresponds to the polyphenol compounds

Table 4. Operating Conditions for Overall Scheme

	Cleaning and Preconcentration Stage	
adsorbent	C18 (500 mg) solvated with 10 mL of methanol conditioned with 3 mL of the saturated NaCl solution at a pH of 2 (25 g of NaCl in 100 mL of 0.01 M HCl)	
sample	5 mL of wine is diluted with 5 mL of a saturated solution of NaCl at a pH of 2 (25 g of NaCl in 100 mL of 0.01 M HCl)	
washing solvent	1°: 1 mL of the saturated NaCl solution at a pH of 2 (25 g of NaCl in 100 mL of 0.01 M HCl) 2°: 1 mL of a solution of 0.01 M HCl	
elution	the analytes retained in the adsorbent are eluted with 0.5 mL of methanol	this eluate corresponds to the polyphenol compounds
	Fractionation Stage	
adsorbent	SAX (500 mg) equilibrated with 10 mL of water	
sample	to the eluate previously obtained a volume of 2.5 mL of a buffered phosphate solution of pH = 6.5 and $I = 0.05$ M is added	the eluate in which the neutral polyphenols should be present is collected in this step
washing solvent	1 mL of water	
elution	1 mL of 1 M HCl	this eluate corresponds to the acid polyphenol fraction

Table 5. Study of the Reproducibility of the SPE Sample Preparation Method

compd	recovery (%)	RSD (%)
gallic acid	5.0	19.6
protocatechuic acid	20.7	10.9
protocatequialdehyde	17.8	12.6
gentisic acid	51.1	6.3
p-hydroxybenzoic acid	53.4	10.0
catechin	51.9	7.4
p-hydroxybenzaldehyde	52.0	7.3
m-hydroxybenzoic acid	80.9	7.9
vanillic acid	78.5	12.9
caffeic acid	72.3	11.9
syringic acid	81.8	11.7
vanillin	57.8	7.3
epicatechin	58.0	7.3
syringialdehyde	67.6	1.9
p-coumaric acid	74.8	13.2
o-vanillin	58.5	2.9
ferulic acid	71.6	11.7
veratric acid	71.6	11.7
sinapic acid	45.5	22.7
o-coumaric acid	66.2	22.5
3,4,5-trimethoxycinnamic acid	55.4	15.6
3,5-dimethoxybenzaldehyde	62.3	4.2

permit the identification of 12 acid and seven neutral species. The technique produces chromatograms which are easier to identify than those obtained from the use of other sample preparation techniques not involving fractionation, since there are present a minority of

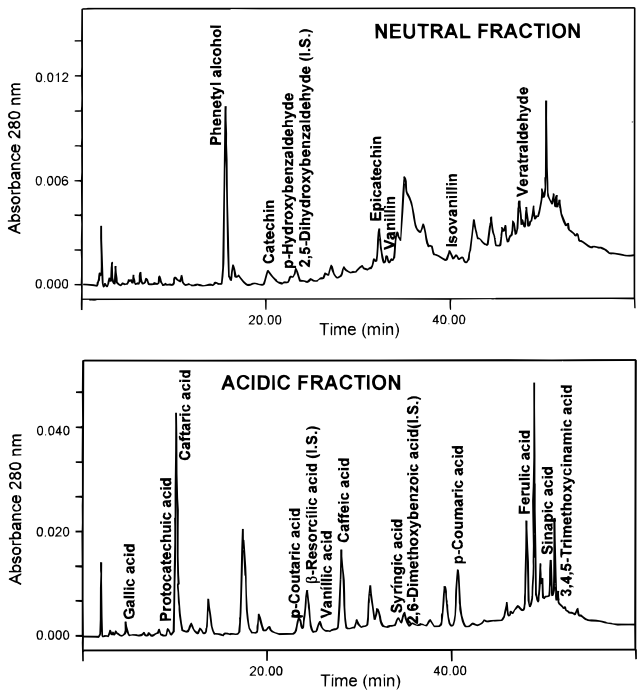

Figure 1. Chromatograms (280 nm) of the fractions collected after application of the SPE sample preparation method to samples of a "fino" sherry wine.

species which are coeluted with others and, thus, cannot be detected.

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